Dipyridamole Potentiates the Growth-Inhibitory Action of Methotrexate and 5-Fluorouracil in Human Keratinocytes In Vitro

Pauline M. Schwartz, Ph.D., and Leonard M. Milstone, M.D.

Dermatology Service, VA Medical Center, West Haven, and Department of Dermatology, Yale University School of Medicine, New Haven, Connecticut, U.S.A.

Human keratinocytes transport extracellular thymidine across the plasma membrane and incorporate it into DNA. Data presented here show that dipyridamole, a well-known inhibitor of facilitated diffusion of nucleosides, blocks the transport of thymidine into human keratinocytes in vitro. Dipyridamole (1.0 μM) inhibited the transport of 3H-thymidine (0.2 μM) into intracellular material by 75% and its subsequent salvage and incorporation into DNA by 48%.

Dipyridamole (1 μM) did not affect the growth of keratinocytes in vitro but did potentiate the growth inhibition caused by methotrexate (MTX) or 5-fluorouracil (5-FU). The growth of keratinocytes exposed to 0.1 μM MTX for 8 d was inhibited by 32%. However, in combination with a non-inhibitory concentration of dipyridamole (1 μM), this concentration of MTX (0.01 μM) inhibited the growth of keratinocytes by 93%. Thymidine in culture medium reversed the cytotoxicity of MTX. However, in the presence of dipyridamole, thymidine in the culture medium did not reverse the action of MTX. The synergistic interaction between MTX and dipyridamole was also observed with 5-FU and dipyridamole. 5-FU (0.5 μM) inhibited cell growth by 30% but in combination with dipyridamole (1 μM), inhibited cell growth by 86%. These data are consistent with the theory that inhibiting thymidine salvage by blocking transport of extracellular thymidine potentiates the growth inhibitory action of inhibitors of de novo pyrimidine biosynthesis in human keratinocytes. Combination chemotherapy, such as methotrexate plus dipyridamole, might be efficacious in the treatment of hyperproliferative diseases of the epidermis.
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The normal epidermis is a continually renewing, stratified epithelial tissue containing proliferating keratinocytes that are located primarily in the basal layer [1–3]. The proliferating keratinocytes synthesize DNA and, among other requirements, need a supply of the four deoxynucleoside triphosphates, which are the substrates for DNA polymerase [4,5]. Thymidine triphosphate (dTTP) is one of these essential precursors. The replicating keratinocyte, like other proliferating mammalian cells, can synthesize dTTP by two possible routes (Fig 1) [4]. The cell can synthesize dTMP from amino acids, CO_2, and ATP via a 10-step route denoted as the de novo pyrimidine pathway. dTMP can then be phosphorylated in two steps to dTTP. Alternatively, the cell can synthesize dTMP via the salvage pathway in which thymidine, derived from extracellular sources, is phosphorylated by thymidine kinase. There is ample evidence that keratinocytes are capable of both de novo and salvage synthesis of dTTP [6–8].

The salvage of thymidine commences with the uptake of extracellular thymidine into the cell. In many cell types, extracellular thymidine is transported by a facilitated diffusion mechanism that can be blocked by nucleoside transport inhibitors [9]. Nucleoside transport inhibitors are a class of chemically diverse structures, and the mechanisms by which they act have been well studied [9–12]. Nucleoside transport inhibitors include dipyridamole, also known as Persantin, dilazep, and nitrobenzylthioinosine [9,10,13]. Dipyridamole and dilazep are used clinically as vasodilators and inhibitors of platelet aggregation, and the action of dipyridamole as a vasodilator is now thought to be due to inhibition of nucleoside transport [14]. Although these transport inhibitors interfere with the salvage of nucleosides such as thymidine, they are not potent inhibitors of DNA synthesis presumably because cells utilize de novo pathways to make necessary nucleic acid precursors.

The use of dipyridamole to block the transport and subsequent utilization of thymidine has been explored recently in a number of combination chemotherapeutic regimens to treat malignant neoplastic diseases. These combination chemotherapies have been designed to simultaneously block both the salvage and de novo pathways leading to the synthesis of dTTP by using a nucleoside transport inhibitor in combination with an inhibitor of de novo pyrimidine biosynthesis [15]. One reason to expect a synergistic interaction is that the growth inhibitory activity of many inhibitors of the de novo pyrimidine pathway is reduced by the presence of thymidine, which rescues cells by circumventing the blockade of

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Reprint requests to: Pauline M. Schwartz, Ph.D., VA Medical Center, West Spring Street, West Haven, CT 06516.

Abbreviations:

DMEM: Dulbecco's Modified Eagle's Medium
dTMP: thymidine 5′-monophosphate
dTTP: thymidine 5′-triphosphate
EDTA: ethylenediaminetetraacetic acid
MTX: methotrexate
PBS: phosphate-buffered saline
PCA: perchloric acid
TCA: trichloroacetic acid
5-FU: 5-fluorouracil

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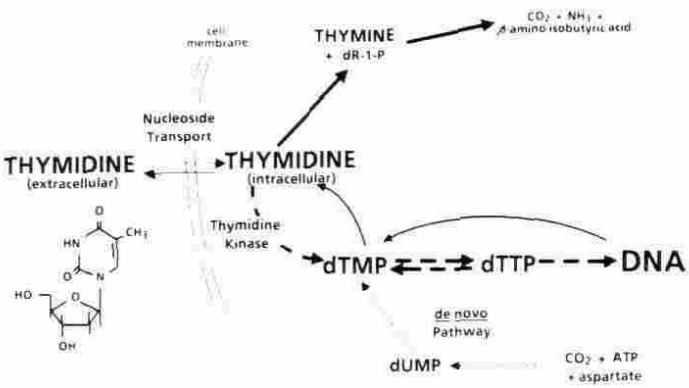

Figure 1. Biochemical Pathways of Thymidine Metabolism. Cells can synthesize dTMP via the de novo pathway *(dotted arrow)* or by the salvage pathway by which thymidine is transported into the cell *(dashed, two-way arrow)* and phosphorylated by thymidine kinase *(dashed one-way arrow)*. dTMP can be converted in two steps to dTTP, which is one of four deoxynucleoside triphosphates needed for DNA synthesis. Abbreviations: *dUMP*, 3′-de-oxyuridine 5′-monophosphate; *dR-1-P*, deoxyribose-1-phosphate; *dTMP*, thymidine 5′-monophosphate; *dTTP*, thymidine 5′-triphosphate.

the de novo pyrimidine pathway [16,17]. A number of such combinations have been tested in vitro. Such studies show that nucleoside transport inhibitors, such as dipyridamole, can potentiate the growth inhibitory action of inhibitors of de novo pyrimidine biosynthesis [15,18–21].

The use of nucleoside transport inhibitors has relevance in vivo because the circulation contains a low but sustained amount of thymidine available for salvage. Indeed, thymidine in the circulation has been shown to modulate the action of inhibitors such as methotrexate in vivo [16]. Recently, clinical trials were initiated to determine the efficacy of such combinations as MTX plus dipyridamole [22,23] and 5-FU plus dipyridamole [24] for treatment of neoplastic diseases.

The epidermis is subject to a number of hyperplastic diseases that are treated with inhibitors of DNA synthesis such as MTX and 5-FU [25,28]. As one step in a rational approach to combination chemotherapy for dermatologic diseases, we undertook a study of dipyridamole to determine if it acted as a nucleoside transport inhibitor in human keratinocytes and if dipyridamole would potentiate the cytotoxic activity of MTX and 5-FU in proliferating keratinocytes in vitro.

METHODS

Primary Human Keratinocytes Cell Cultures Primary cultures of human neonatal foreskin keratinocytes were grown on a feeder layer of irradiated 3T3 cells as previously described [3]. Primary cultures were maintained in complete medium consisting of Dulbecco's Modified Eagle's Medium supplemented with 20% fetal bovine serum, hydrocortisone (0.4 μg/ml), epidermal growth factor (10 ng/ml), insulin (10 μg/ml), cholera toxin (100 pM), penicillin (100 U/ml), and streptomycin (100 μg/ml). The Ca^{++} concentration was 1.8 mM. Preconfluent, stratified cultures were washed with EDTA (0.2 g/L Hanks' balanced salt solution) to remove fibroblasts and then with trypsin-EDTA (0.5 g trypsin and 0.2 g EDTA /L in Hanks' balanced salt solution) to remove keratinocytes.

Uptake of 3H-Thymidine into Keratinocytes Keratinocytes from a primary culture were suspended in DMEM at 4×10^6 cells/ml. The keratinocytes were shaken in a water bath at 37° in medium with or without dipyridamole. After 20 min, 3H-thymidine (0.2 μM, 4 μCi/ml) was added. Ten minutes after the addition of 3H-thymidine, a 100 μl sample of cell suspension was layered on top of a 400 μl microfuge tube containing 100 μl of oil (Paraffin: Silicon, 16:84) on top of 75 μl of 3N PCA. The tube was immediately centrifuged (13,000 rpm for 1 min) and then frozen at −70°. The middle oil layer separates the upper layer, containing culture medium, from the bottom layer, containing cells lysed by PCA [29]. The frozen tube was cut through the oil partition and the upper and lower portions placed in separate scintillation vials and counted. Radioactivity in the PCA layer was taken as a measure of the intracellular uptake of thymidine. The nature of the intracellular metabolites of thymidine formed in 10 min was not determined.

Incorporation of 3H-Thymidine into DNA of Human Keratinocytes In Vitro Secondary cultures of keratinocytes were prepared in 35-mm dishes containing a feeder layer of irradiated 3T3 cells. Well-stratified cultures were used 10–14 d after reaching confluence. Cultures of human keratinocytes were fed with 0.8 ml of DMEM alone or DMEM containing various concentrations of dipyridamole. After 1 h, 200 μl of 3H-thymidine was added to give a final concentration of 0.2 μM thymidine, 4 μCi/ml. After 2 h culture dishes were washed with PBS and then with cold 10% TCA to remove unincorporated radioactivity. The cells were hydrolyzed with 3% PCA at 90° for 30 min. The PCA extract was transferred to a scintillation vial. Incorporation of thymidine into DNA is taken to be the radioactivity in the TCA-insoluble PCA-hydrolyzable material [30].

Growth Studies For growth studies, secondary cultures of keratinocytes were prepared by plating 2×10^4 keratinocytes into 35-mm dishes with complete MCDB-153. Complete MCDB-153 is basal medium supplemented with epidermal growth factor (5 ng/ml), insulin (5 μg/ml), hydrocortisone (0.4 μg/ml), ethanolamine (0.01 mM), phosphoethanolamine (0.01 mM), transferrin (10 μg/ml), bovine pituitary extract (50 μg protein/ml), and additional amino acids [histidine (2.4×10^{-4}M), isoleucine (7.5×10^{-4}M), methionine (9×10^{-5}M), phenylalanine (9×10^{-5}M), tryptophan (4.5×10^{-5}M), and tyrosine (7.5×10^{-5}M)]. The Ca^{++} concentration is 0.1 mM. Basal MCDB-153 contains 3 μM thymidine. MCDB-153 without thymidine was formulated by Clonetics, Inc. (Boulder, CO). Cultures were fed with control and test media starting the day after the cells were seeded. Cultures were fed every 2 to 3 d with control and test media for 8 d. The cells in culture dishes were enumerated, after trypsinization, in a Coulter counter or were stained with Rhodanile blue.

Chemicals DMEM and sterile tissue culture solutions were purchased from Gibco Laboratories (Grand Island, NY). Fetal bovine serum was purchased from JR Scientific (Woodland, CA). Basal MCDB-153 was obtained from Irvine Scientific (Santa Ana, CA), and MCDB-153 without thymidine was purchased from Clonetics, Inc. (Boulder, CO). 3H-Thymidine (20Ci/mmole) was purchased from New England Nuclear (Boston, MA). Methotrexate, parenteral (Lederle Parenterals, Carolina, Puerto Rico), was reconstituted with sterile water and diluted in culture medium for experimental use. Dipyridamole and 5-fluorouracil were purchased from Sigma Chemical Co. (St. Louis, MO); stock solutions were prepared in culture medium and sterilized by filtration. Piritrexim was the gen-

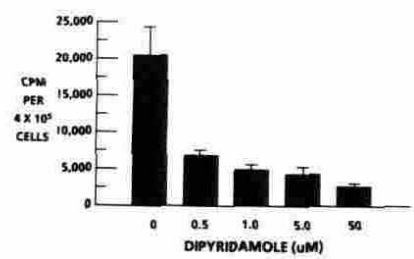

Figure 2. Dipyridamole Reduces Uptake of 3H-Thymidine into Human Keratinocytes. 3H-Thymidine (0.2 μM, 4 μCi/ml) was added to a suspension of keratinocytes in the presence or absence of dipyridamole. After 10 min, cells were separated from the extracellular medium by centrifugation through a layer of oil. Cell-associated radioactivity was measured. The mean and range of data from a representative experiment are shown.

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RESULTS

Effect of Dipyridamole on the Uptake of 3H-Thymidine into Keratinocytes The uptake of 3H-thymidine (0.2 μM, 4 μCi/ml) into keratinocytes was rapid and reached a plateau within 5 min (data not shown). After 10 min, intracellular thymidine was less than 5% of the extracellular thymidine. Dipyridamole (1 μM) inhibited the uptake of 3H-thymidine by more than 75% compared to control cells (Fig 2). The effect of dipyridamole on the uptake of 3H-thymidine was dose dependent.

Effect of Dipyridamole on the Incorporation of 3H-Thymidine into DNA of Keratinocytes Confluent, stratified cultures of human keratinocytes incorporated 3H-thymidine (0.2 μM, 4 μCi/ml) into DNA (Fig 3). Dipyridamole inhibited incorporation of 3H-thymidine into DNA of keratinocytes in a dose-dependent fashion. A 50% inhibition of 3H-thymidine incorporation into DNA was seen when cells were exposed to 1 μM dipyridamole starting 1 h before the thymidine pulse.

Effect of Dipyridamole and Methotrexate on the Growth of Keratinocytes Cultures of keratinocytes grew to $5.5 - 8 \times 10^5$ cells per dish in 8 d after seeding at 2×10^4 cells per dish and feeding with complete MCDB-153 (Fig 4). Dipyridamole at 1 μM in the medium did not affect the growth of keratinocytes but 3 μM dipyridamole did inhibit cell growth. The effect of MTX alone on the growth of keratinocytes was dose dependent: 0.05 μM MTX had no effect on cell growth, but 0.5 μM MTX inhibited cell growth by approximately 70%. A non-inhibitory concentration of dipyridamole (1 μM) increased the inhibitory effect of MTX substantially. MTX (0.01 μM) inhibited cell growth by about 30%, but in the presence of 1 μM dipyridamole, inhibited cell growth by over 90%. A 0.05 μM dose of MTX alone was non-inhibitory but inhibited cell growth by about 20% in the presence of 1 μM dipyridamole.

Effect of Thymidine on the Interaction of Dipyridamole with Methotrexate The previous growth experiments were conducted in complete MCDB-153 formulated with 3 μM thymidine [31]. Thymidine is known to reverse the cytotoxic activity of MTX in a number of cell lines [16,17]. The influence of thymidine on the action of dipyridamole (1 μM), methotrexate (0.1 μM), and a combination of these agents was measured. The concentration of thymidine (3 μM) normally found in MCDB-153 had no affect on keratinocyte growth in the absence (Fig 5*a*) or in the presence (Fig 5*b*) of dipyridamole (1 μM). However, this same concentration of thymidine nearly reversed the growth inhibition caused by 0.1 μM MTX (Fig 5*c*). The rescue of cells by thymidine from the inhibitory action of MTX was nullified by 1 μM dipyridamole (Fig 5*d*).

Effect of Dipyridamole and 5-Fluorouracil on the Growth of Keratinocytes The growth of keratinocytes was inhibited by about 30% when cells were grown in the presence of 0.5 μM 5-FU (Fig 6). In the presence of 1 μM dipyridamole, 0.5 μM 5-FU inhibited cell growth by almost 85%. The effect of 1 μM 5-FU also was enhanced by 1 μM dipyridamole.

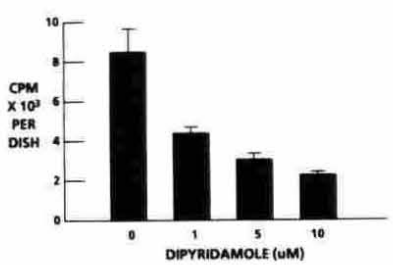

Figure 3. Dipyridamole Reduces Incorporation of 3H-Thymidine into DNA of Keratinocytes. After a 1-h pre-exposure of cultures to dipyridamole, 3H-thymidine (0.2 μM, 4 μCi/ml) was added for 2 h. The radioactivity in the TCA-insoluble, PCA-hydrolyzable fraction was measured. The mean and range of data from a representative experiment are shown.

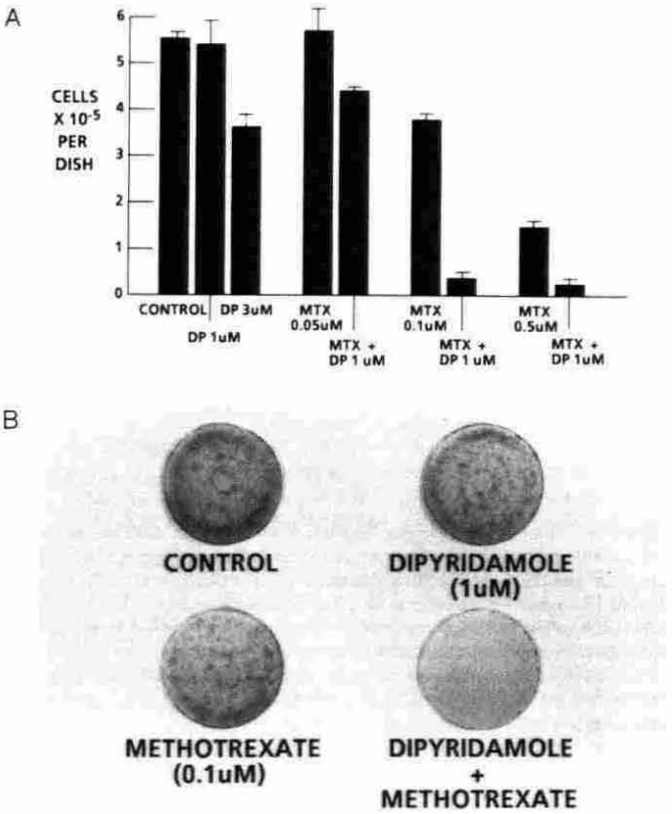

Figure 4. Dipyridamole and Methotrexate Synergistically Inhibit the Growth of Human Keratinocytes in vitro. Cultures of keratinocytes were fed the day after seeding with medium containing dipyridamole, methotrexate, or dipyridamole plus methotrexate; control cultures were fed medium containing no drugs. Cultures were fed every 2 to 3 d with control or test medium for 8 d. The number of cells per dish was determined using a Coulter Coulter *(A)*. The mean and range of data from a representative experiment is shown. Representative cultures were stained with Rhondanile blue *(B)*. Culture dishes showed a diffuse staining typical of pre-confluent cultures grown in complete MCDB containing 0.1 mM Ca^{++}. Abbreviations: *MTX*, methotrexate; *DP*, dipyridamole

DISCUSSION

Human keratinocytes salvage and utilize extracellular thymidine. The first step in this process is the uptake of thymidine into the cell. Many, but not all, mammalian cells transport nucleosides by facilitated diffusion, which is mediated by a high affinity membrane carrier with broad specificity for nucleosides [9–11,29]. Dipyridamole is an inhibitor of the facilitated diffusion of nucleosides [10,12,15]. Our demonstration that dipyridamole inhibits the uptake of extracellular thymidine into keratinocytes suggests that facilitated diffusion is the major means by which keratinocytes transport thymidine.

Dipyridamole inhibits the transport of thymidine into keratinocytes and thereby prevents the keratinocyte from utilizing an extracellular source of thymidine to synthesize thymidine nucleotides and DNA. Dipyridamole (1 μM) did not inhibit the proliferation of keratinocytes exposed to the drug for 8 d, although this concentration of dipyridamole would be expected to give a significant, but not complete, inhibition of thymidine transport. These observations with dipyridamole suggest that proliferation of keratinocytes in vitro may not depend on the availability of thymidine in the culture medium. In addition, keratinocytes grew as well in the culture me-

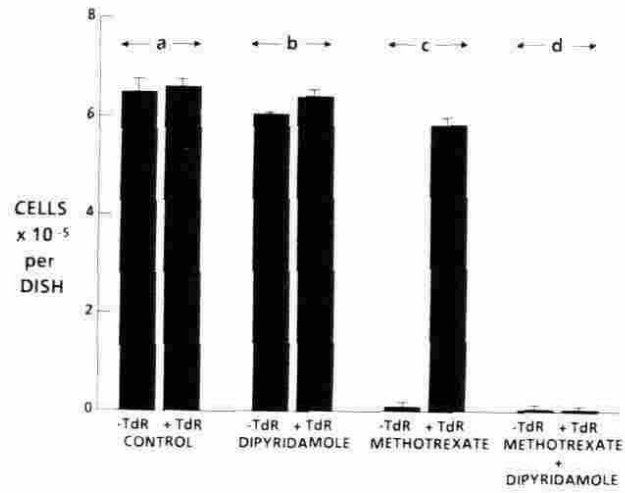

Figure 5. Thymidine Reverses Growth Inhibition by Methotrexate, but not in the Presence of Dipyridamole. Cultures of keratinocytes were fed the day after seeding with medium containing dipyridamole (1 μM), methotrexate (0.1 μM), or dipyridamole plus methotrexate in medium without thymidine or with 3 μM thymidine. Control cultures were fed medium with or without thymidine-containing no drugs. Cultures were fed every 2 to 3 d with control or test medium for 8 d. The number of cells per dish was determined using a Coulter Counter. The mean and range of data from a representative experiment are shown.

dium without thymidine as in medium containing thymidine. Thus, rapidly growing keratinocytes must make use of de novo pathways to synthesize sufficient thymidine nucleotides to sustain growth.

Dipyridamole at a concentration of 3 μM moderately inhibited keratinocyte replication. In other in vitro systems, dipyridamole inhibited cell growth at concentrations that were 3 to 10 times greater than the concentration producing significant inhibition of thymidine uptake [15,21]. These high concentrations of dipyridamole may inhibit growth by producing an imbalance in the concentrations of all intracellular nucleotides as a result of the inhibition on transport of nucleosides both into and out of the cell.

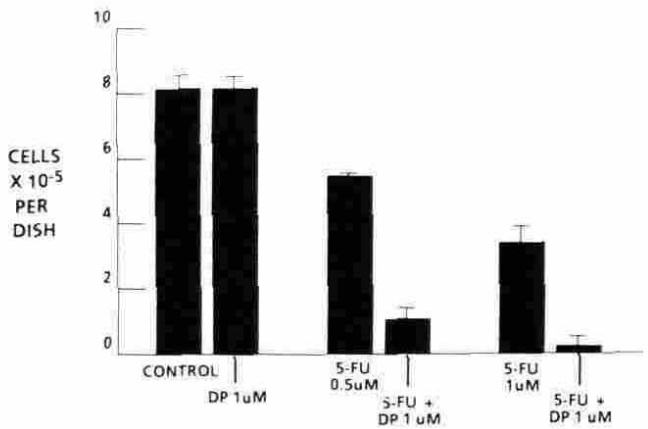

Figure 6. Dipyridamole and 5—Fluorouracil Synergistically Inhibit the Growth of Human Keratinocytes in vitro. Cultures of keratinocytes were fed the day after seeding with medium containing dipyridamole, 5-fluorouracil, or dipyridamole plus 5-fluorouracil; control cultures were fed medium containing no drugs. Cultures were fed every 2 to 3 d with control or test medium for 8 d. The number of cells per dish was determined using a Coulter Counter. The mean and range of data from a representative experiment are shown.

Methotrexate inhibits the growth of rapidly proliferating human keratinocytes in vitro and in vivo by inhibiting de novo pyrimidine biosynthesis [26,28,32]. It could be predicted that this action of MTX might be circumvented by providing an alternative source of pyrimidines. We have shown that in vitro, MTX was most effective in the absence of thymidine, and its action was reversed by the addition of thymidine. Similarly, Flaxman et al demonstrated that keratinocytes treated with a growth-inhibitory concentration of MTX paradoxically incorporated a greater amount of 3H-thymidine than did control cells [32]. Presumably, cells treated with MTX rapidly salvage thymidine to overcome the depletion of dTTP derived from de novo pathways. Our findings suggest that previously reported variability in sensitivity of epidermal cells in vitro to MTX [33] may be due in part to the presence and utilization of extracellular thymidine.

In contrast to our demonstration that exogenous thymidine reverses the action of MTX, dipyridamole potentiates the cytotoxic action of MTX in human keratinocytes in vitro. This interaction with dipyridamole is not limited to MTX. Dipyridamole also potentiated the growth-inhibitory activity of piritrexim, a new lipophilic antifolate with activity against psoriasis [34] (H. Reuveni, personal communication) and 5-FU, a pyrimidine antimetabolite, in human keratinocytes in vitro. In other experimental systems, this interaction between dipyridamole and inhibitors of pyrimidine biosynthesis has been shown to be due to the simultaneous block of both salvage and de novo pathways to the formation of thymidine nucleotides [15,20,21]. The ability of thymidine to reverse the action of MTX and the ability of dipyridamole to potentiate the action of MTX substantiates this simultaneous blockade as the mechanism by which the combination of MTX and dipyridamole acts synergistically in keratinocytes. Moreover, it provides further evidence that human keratinocytes in vitro possess and utilize both salvage and de novo pathways for the biosynthesis of dTMP. The mechanism by which dipyridamole potentiates the action of 5-fluorouracil in keratinocytes was not explored. In other cell types this interaction has been attributed to the blockade of thymidylate synthase by FdUMP concurrently with either inhibition of the salvage of thymidine or the intracellular accumulation of FdUMP because it is trapped inside the cell by the nucleoside transport inhibitor [18].

The systemic use of dipyridamole in combination with MTX, 5-FU, or other agents is being tested in several malignancies [22–24]. The effective use of this combination therapy for wide-spread diseases such as cancer may be limited by a concommitant increase in toxicity to normal cells. Moreover, extensive binding of dipyridamole to plasma proteins after systemic administration of the drug may make it impossible to achieve a plasma concentration of dipyridamole that will be sufficient to block thymidine salvage in tissues [35]. Combinations of agents such as dipyridamole and methotrexate or dipyridamole and 5-fluorouracil could be more effective and less toxic when it is possible to apply at least one of these agents topically. Several dermatologic diseases may be responsive to such a combination chemotherapeutic approach.

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